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14. ABSTRACT (technical abstract)

This proposal addresses the myeloproliferative disorders, as well as the blood cancers FY14 PRCRP topic areas. It is responsive to the following Military Relevance Focus Area: Gaps in cancer prevention, diagnosis, early detection, or treatment that may affect the general population but have a particularly profound impact on military health with a special focus on cancers associated with conditions, or circumstances, disproportionately represented within the military beneficiary population. Exposure to radiation, chemical and environmental carcinogens may be contributing factors to these blood diseases.

Calreticulin (CALR) is known to be a major player in the ER quality control of glycosylated proteins. Remarkably, this ubiquitously expressed housekeeping gene is mutated in ~30% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), 2 of the 8 major myeloproliferative neoplasms (MPNs) as classified by the world health organization. One of the biggest challenges is to understand how Jak2, MPL and CALR mutant proteins can lead to very similar signaling events and phenotypes in these MPNs.

Objective/Hypothesis. We hypothesize that ET and PMF phenotypes result from abnormal signaling and aberrant intracellular trafficking events in cells bearing mutations in *JAK2*, *MPL* or *CALR*. In this project, we will acquire a deeper understanding of the interplay between these three gene products. We will compare and contrast intracellular routing for wildtype and mutated proteins, identify their potential intersections, and determine the cellular locations from which signals are propagated. Our work seeks a broader understanding of the pathogenesis of the MPNs and set the stage for development of targeted therapeutic solutions. Our **Specific Aims** are:

Aim 1. To determine the sub-cellular localization of mutant calreticulin, as well as the impact on MPL trafficking and megakaryocyte (MK) ultrastructure.

Aim 2. To evaluate mechanisms underlying aberrant signaling in MPNs bearing mutant CALR or MPL.

Study Design. In Aim 1, we will map the spatial distributions of calreticulin and MPL using high resolution microscopy techniques, genetically engineered cell lines and bone marrow megakaryocytes from ET and PMF patients. We will use CRISPR editing to mutate one or both *CALR* alleles in megakaryocytes, add fluorescent tags, and study effects on overall cell ultrastructure during proplatelet biogenesis. Novel correlated light-EM (electron microscopy) methods will determine the precise subcellular localization of mutant calreticulin and Mpl, coupled to 3D EM tomography for mapping the inner membrane system and location of these proteins. The impact of mutant calreticulin on MPL trafficking will be determined, using western blotting, pull-down and proximity ligation assays. The genetically engineered human megakaryocyte progenitor cell lines will be used to engraft immunocompromised mice, for *in vivo* analysis of proliferation and differentiation. To confirm clinical relevance of these findings, the levels and cellular location of MPL, calreticulin and Jak2 proteins in primary megakaryocytes will be compared in samples from normal individuals and MPN patients with *CALR* mutations. Profiling of MPL glycosylation status in human platelets will be based on western blotting analysis.

Aim 2 will focus on the impact of calreticulin mutants on signal transduction. We will apply powerful quantitative assays, including live cell imaging and a novel proteomics approach based on the APEX2-tag biotinylation proximity labeling assay. The state of the ER calcium store will be determined using a FRET-based biosensor. In addition, thrombopoietin (TPO)-stimulated calcium mobilization will be evaluated in megakaryocyte cell lines expressing WT and mutant calreticulin using ratio imaging methods. Using both genetically engineered culture cells and cells recovered from the xenograft model, we will also evaluate readouts of the MPL-Jak/STAT pathway in presence, or absence, of exogenous TPO or when MPL is trapped in intracellular compartments where ligand-independent dimerization may be occurring.

15. SUBJECT TERMS

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Table of Contents

	Page
1. Introduction……	2
2. Keywords……	2
3. Accomplishments……	2
4. Impact……	4
5. Changes/Problems..……	5
6. Products……..	7
7. Participants & Other Collaborating Organizations……..	7
8. Special Reporting Requirements……	10
9. Appendices……	11

1. Introduction

Calreticulin (CALR) is known to be a major player in the ER quality control of glycosylated proteins. Remarkably, this ubiquitously expressed housekeeping gene is mutated in ~30% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), two of the 8 major myeloproliferative neoplasms (MPNs) as classified by the WHO. One of the biggest challenges is to understand how Jak2, MPL and CALR mutant proteins can lead to very similar signaling events and phenotypes in these MPNs. We hypothesize that ET and PMF phenotypes result from abnormal signaling and aberrant intracellular trafficking events in cells bearing mutations in *JAK2, MPL* or *CALR*. In this project, we will acquire a deeper understanding of the interplay between these three gene products. We will compare and contrast intracellular routing for wildtype and mutated proteins, identify their potential intersections, and determine the cellular locations from which signals are propagated. Our work seeks a broader understanding of the pathogenesis of the MPNs and set the stage for development of targeted therapeutic solutions.

2. Keywords

- Thrombopoietin Receptor (Mpl)
- Janus kinase 2 (Jak2)
- Calreticulin (CALR)
- Myeloproliferative Neoplasms (MPN)
- Essential Thrombocythemia (ET)
- Congenital Amegakaryocytic Thrombocytopenia (CAMT)

3. Accomplishments

a. Major Goals.

- To determine the sub-cellular localization of mutant calreticulin, as well as the impact on MPL trafficking and megakaryocyte (MK) ultrastructure.
- To evaluate mechanisms underlying aberrant signaling in MPNs bearing mutant CALR or MPL.

b. Accomplishments during this reporting period

Establishing the UT-7/Tpo sub-line. In order to establish the UT-7/Tpo sub-line, the UT-7 cells have been grown at sub-optimal concentrations of thrombopoietin (Tpo) and in the absence of other cytokines. Expression of Mpl in the Tpo-selected cells was assessed by western blotting (WB). This new cell line has then been sub-cloned and individual clones were screened for robust Mpl expression using WB before being further modified (**Fig. 1**).

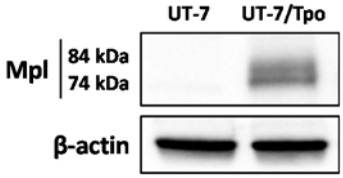

Figure 1. Mpl expression level in UT-7 and Tpo-selected UT-7 cells, UT-7/Tpo.

CRISPR-engineering of UT-7/Tpo cells. Our first goal has been to introduce the most common calreticulin (CRT) mutation (Type 1 or T1) found in MPNs into UT-7/Tpo cells using genetic engineering. The left panel in **Fig.2** show the presence of a sub-population of UT-7/Tpo cells that express the T1 mutant form of calreticulin after the genome editing experiment. Sub-cloning of these cells allowed us to identify edited cells that are homozygous for this mutation, as shown by PCR screening shown in the right panel of **Fig. 2**. Further editing of these cells

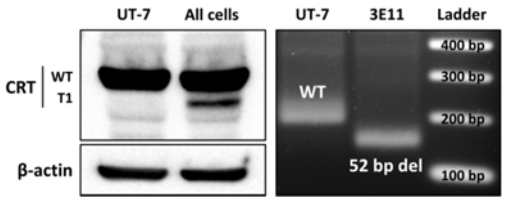

Figure 2. Introduction of calreticulin T1 mutation in UT-7/Tpo cells using genome engineering.

in order to establish a unique model system for MPN studies to introduce fluorescent tags for calreticulin and/or Mpl are ongoing.

Studies of a novel Double Mpl Mutation. It is important to note that, while activating mutations in these genes result in thrombocytosis (too *many* platelets), there are also distinct mutations that result in thrombocytopenia (too *few* platelets). During this initial reporting period, we were able to study a new double Mpl mutation that we discovered (in collaboration with our colleagues in Nimes, France). In this case report of 3 siblings with congenital amegakaryocytic thrombocytopenia (CAMT), we show that the Mpl gene had two mutations in tandem – both an activating mutation (Mpl K39N) and an inactivating mutation (W272R). Because of three children in this family had severely low platelet function (including one fatality), this was an amazing opportunity to study how the inactivating mutation was able to override the gain-of-function mutation. We used our powerful tools for imaging and genome editing to study the intracellular trafficking of both singly- and doubly-mutated Mpl, fitting well with Aim 2 of this project.

Highlights from the new manuscript showed this novel mutation results in entrapment of the mutant Mpl in the ER, where it is co-localized with wildtype CALR. Thus, aberrant trafficking of Mpl is responsible for the absence of proper signaling and thus, the phenotype of severe thrombocytopenia in affected patients

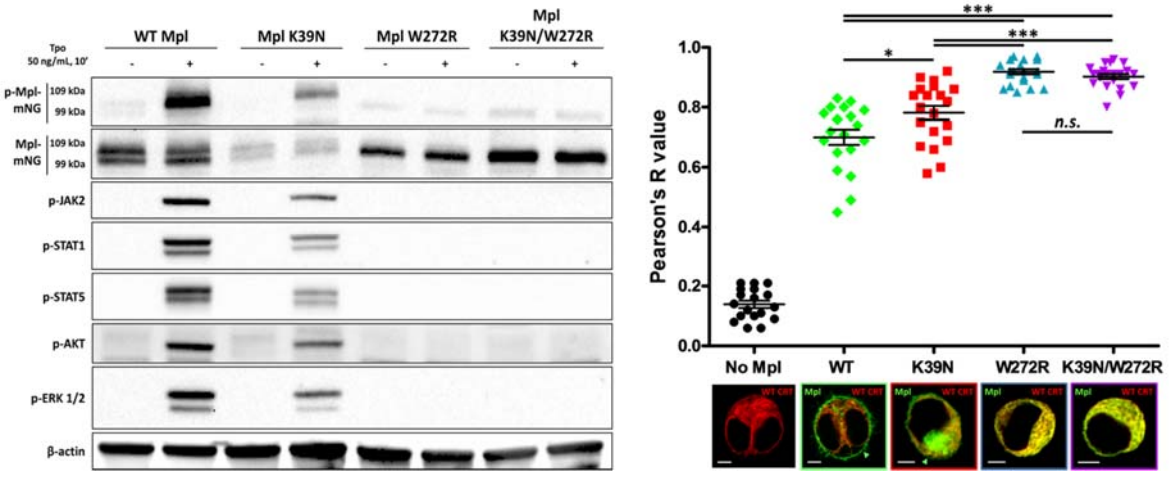

Figure 3. Aberrant intracellular trafficking of Mpl is responsible for absence of response to its ligand, Tpo, in UT-7 cell lines. At left, we evaluated Tpo signaling through the JAK/STAT, MAPK and PI3K pathways. Both WT and K39N-mutated Mpl were competent for signaling, while single or double mutants bearing W272R were unresponsive to Tpo. At right, Co-expression of WT CALR fused to RFP (ER marker) showed significantly higher co-localization (R value) with mutant Mpl than with WT Mpl, evidence that most receptors were retained within the ER.

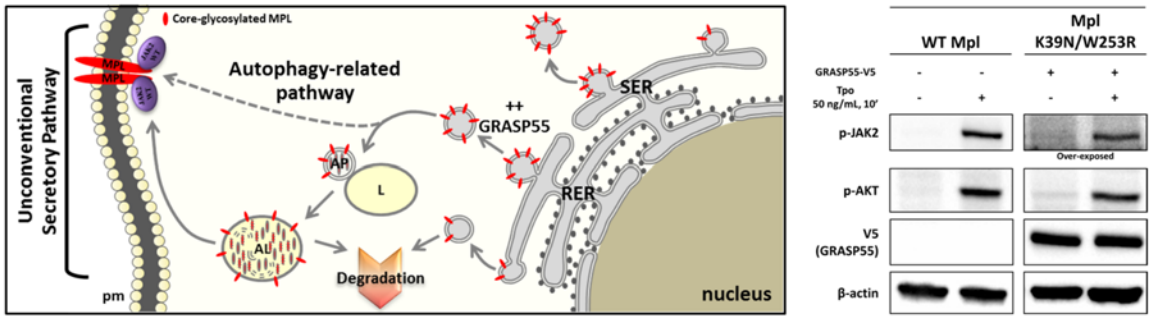

Figure 4. Tpo-induced signaling was partially restored via GRASP55 over-expression, forcing ER-trapped Mpl to traffic to the cell surface using unconventional secretion. Left panel adapted from Cleyrat *et al.* Traffic 2014.

(**Fig. 3**). Preliminary data on this CAMT family was provided in the grant application and we are very pleased that this study could be initiated and completed during the first reporting period.

Additional data from this new manuscript are shown in **Figures 4-5,** addressing our stated goal to develop new targeted therapeutic solutions. Our goal was to establish "proof-of-principle" for approaches that might functionally rescue the mutated thrombopoietin receptor. We previously showed that an autophagy-dependent, "alternative" secretory pathway can allow the ER form of Mpl to reach the plasma membrane. By overexpressing GRASP55, we showed that activation of this secondary trafficking pathway was successful in rescuing part of Mpl function and response to Tpo (**Fig. 4**).

Results were even more striking when using genome editing to restore the wildtype sequence of the gene coding for Mpl in order to fully rescue its function and response to its ligand (**Fig. 5**). Genetic editing (using CRISPR/Cas9) performed on cells carrying the W272R mutation restored the WT sequence and the response to Tpo ([Tpo] = 3 ng/mL), with similar cell proliferation as WT Mpl cells.

Thus far, our study of a newly discovered mutation of Mpl (W272R) that effects the trafficking and signaling of Mpl has led to important findings on Mpl sub-cellular trafficking and its impact on basic cell signaling. Function of the deficient Mpl receptor could be rescued using two separate approaches: CRISPR/Cas9 genetic engineering and GRASP55 over-expression. These results add to our understanding of Mpl multiple roles in the pathogenic mechanisms of several myeloid malignancies and significantly increase the relevance of our upcoming experiments using the CRISPR-edited UT-7/Tpo cell lines.

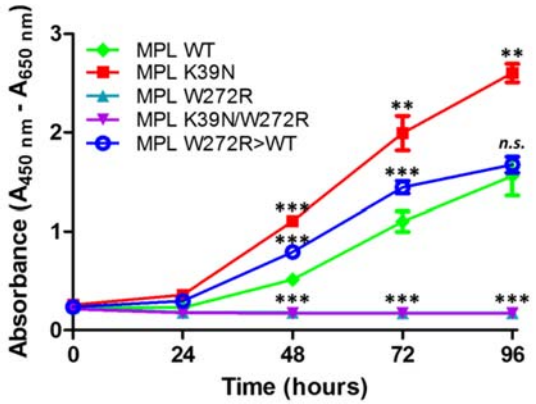

Figure 5. Functional rescue of mutant Mpl (Mpl W272R>WT) using genome editing.

Establishing a pipeline of MPN patient samples. Our IRB-approved protocol (HRRC#05-435) to consent and collect blood and bone marrow from MPN patients opened in January 2016. Enrollment has been excellent, with 45 samples collected and processed. We have refined our methods for generating megakaryocytic colonies from progenitor cells in these samples. The most common MPN mutations are well represented in this cohort of patients thus far.

c. Opportunities for training and professional development provided by the project - *Nothing to report*

d. Dissemination to communities of interest
   - Press release issued by the UNM Cancer Center generated an article in the Albuquerque Journal, as well as a local TV interview at http://cancer.unm.edu/newsroom/new-grant-helps-new-mexicans-with-blood-disorders/

e. Goals and Objectives for the next reporting period
   - Study CRISPR-derived CALR and Mpl mutants by novel imaging approaches, including 3D reconstruction by EM tomography, as outlined in Aim 1 of the grant proposal.
   - Conduct experiments designed to evaluate the mechanism underlying aberrant signaling in MPNs bearing CALR or Mpl mutants, outlined in Aim 2 of the grant proposal. As described below in Section 5a, revise the experimental plan to respond to new reports that mutant CALR is capable of directly stimulating Mpl-Jak signaling.
   - Complete a case report on a patient enrolled in HRRC#05-435, who progressed from a double mutation status (MPL, CALR) to fatal acute myeloid leukemia (AML). Very interestingly, this Jak2-negative patient was previously enrolled in an early Jak2 inhibitor clinical trial.

4. Impact

a. Impact on the principle disciplinary field - *Nothing to report*
b. Impact on other disciplines - *Nothing to report*
c. Impact on Technology Transfer - *Nothing to report*
d. Impact on society beyond science and technology – *Nothing to report*

5. Changes/Problems

a. Changes in approach and reasons for change

- The MPN field is moving forward rapidly, particularly in the context of CALR mutations. For example, new papers by Araki et al and Marty et al (both in the March 10, 2016 issue of *BLOOD*) demonstrated that the mutant CALR protein specifically activates Mpl. This leads to constitutive activation of the Jak2 pathway. These data from the literature strongly supports our original hypothesis for functional linkage between these three proteins. However, thus far, the location where CALR-Mpl-Jak2 signaling takes place has not been resolved. We will bring all of our tools to bear on this problem. We will also produce recombinant, mutant CALR in order to perform biochemical experiments testing the hypothesis that it acts like a "ligand". We will use our new BLITZ apparatus to evaluate binding to purified Mpl and determine the affinity of this interaction.

  These new reports motivate us to emphasize methods, such as the proximity ligation assay and SuperResolution experiments outlined in the original plan, that will inform us on the intracellular location where CALR-mediated signaling is taking place. Our preliminary data suggests there is little or no secreted CALR, so we expect this location to be the ER. The papers by Araki and Marty also suggest that the planned calcium and APEX experiments are likely to be less informative and may be omitted or minimized in the ambitious experimental plan for next year.

- In the past year, there have also been important papers shedding light on so-called "triple-negative" or sporadic mutations in MPN (reviewed by Harrison and Vannucchi in the January 21, 2016 issue of *BLOOD*). Novel, weak gain-of-function mutations in MPL and Jak2 were discovered using whole exome or next generation sequencing in these patients, as well as rare novel mutations (ie SH2B3). Thus it will be important to fully sequence Mpl, Jak2 (and CALR) for any patients enrolled in HRRC#05-435 whose mutations are not identified by standard screening at diagnosis by the clinical laboratory. If novel mutations are discovered, we will use our battery of assays to determine if they should be classified in the gain-of-function category and if the mutation is associated with abnormal trafficking or constitutive kinase activity.

- The acquisition of additional somatic mutations during disease progression is also of keen interest. With clinical partner, Arana Yi, we have evidence for such a patient. We anticipate completing the sequencing study and plan to prepare a case report for publication.

b. Actual or anticipated problems or delays, with action plan

- Our proposal outlined plans for use of electron microscopy to evaluate the colocalization of calreticulin and MPL, as well as the overall cellular ultrastructure in megakaryocytic cells bearing mutations in these proteins. However, there was a tragic flood in the basement of the Biomedical Research Facility where the electron microscopes were housed. There was a 6-month long process to clean up the facility, evaluate the loss for insurance, and order replacement instruments. Fortunately, the facility is now up and running – with *improved* instrumentation! We will now use a brand new Hitachi HT7700 equipped with an XR16 (16 megapixel) bottom mount

camera for TEM and tilt capabilities for tomography. There is also a new Zeiss Sigma 300 SEM equipped with STEM imaging capabilities, the Atlas 5 workspace, Correlative Array Tomography and a Shuttle and Find system to allow correlative imaging between the Sigma 300 and the Zeiss LSM 800 confocal Airyscan system. The tomography capabilities of the HT7700, in particular, will be applicable in the next reporting period – after a bit of a learning curve to learn the new software. Fortunately, having this equipment at UNM means that we will not have to perform 3D reconstructions at the NCMIR in San Diego, as first proposed in the grant.

- The Animal Model shared resource at the UNM Cancer Center is undergoing a major upgrade in order to meet the new demand for patient-derived xenografts. To take advantage of this opportunity, we delayed implementation of the *in vivo* experiments described in the proposal. We expect to be able to start work on establishing the UT-7 xenograft model in NSG mice, as described in Aim 1, by November 2016. Our amended animal protocol, including the change to include personnel from the Animal Core, is currently under review.

c. Changes with significant impact on expenditures

- Since we now have an electron microscope on site with tomography capabilities, we will reallocate expenses anticipated for use of scopes at the NCMIR in San Diego to biochemical and molecular biology supplies.

- Since the Cancer Center has made considerable investments to upgrade the Animal Model Shared Resource, we will reallocate the 20 percent effort estimated for Dr. Kinjo to pay for charges from the core facility. The new full-time technical lead for the Animal Core, Dr. Irina Lagutina, will inject and monitor mice and invoice us monthly. We expect that the costs will be comparable to our earlier projections – and possibly even with a cost savings which can be applied to biochemical reagents.

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards and/or select agents

- The excellent accrual to HRRC#05-435 prompted us to explore ways to speed up processing time for precious human samples. We have recently made arrangements with the UNM Human Tissue Repository and Tissue Processing Core to receive, process and store samples within hours of their acquisition by the clinical team at the UNM Cancer Center. This professional team is HIPPA compliant and their services (billed monthly) should represent an overall cost savings. This also prevents us from setting up a satellite tissue repository in order to be compliant with new federal regulations. No other research team will have access to these de-identified samples.

- As mentioned in the proposal, use of murine bone marrow-derived megakaryocytes could be an alternative strategy to use of the UT-7 cell line for study of the effect of calreticulin and Mpl mutations on cellular ultrastructure and proplatelet progression. If warranted, this study will require a minor amendment to our existing bone marrow acquisition protocol (USDA Registration #85-R-0014, protocol #14-1001103-HSC).

- Although we maintain our own small colony of triply-deficient NSG (NOD scid gamma) mice (IACUC Protocol #14-101192-B-HSC), we will now have access to mice from the breeding colony managed by the Cancer Center Animal Model core. This is another advantage of the improvements in the Animal Core, ensuring that we will always have sufficient, low-cost mice for xenograft models established by injection of UT-7 cells or primary human bone marrow-derived megakaryocytes.

6. Products

a. Publications, conference papers and presentations

- Dr. Cleyrat was selected for a poster presentation at the American Society for Hematology Annual Meeting (Dec 2015). (see Appendix for abstract)
- Dr. Cleyrat was also selected for a platform presentation at the FASEB Science Research Conference on Genome Editing (June 2016).

- Manuscript submitted (in first stage of review)

  Cédric Cleyrat, Romain Girard, Eun H. Choi, éric Jeziorski, Thierry Lavabre-Bertrand, Sylvie Hermouet, Serge Carillo and Bridget S. Wilson, "Gene Editing Rescue of a Novel MPL Mutant Associated with Congenital Amegakaryocytic Thrombocytopenia" Submitted to *New England J. of Medicine*.

b. Websites/internet sites.

See UNM Cancer Center web pages at
http://hscnews.unm.edu/news/453-000-grant-to-help-unm-researchers-study-blood-disorders
http://cancer.unm.edu/newsroom/unm-cancer-center-scientists-look-inside-cells-to-study-blood-disorders/

c. Technology or techniques.

- Dr. Cleyrat has established techniques for genome editing of human hematopoietic progenitor cells, including use of a multiplex double nickase approach which consists of two separate guide RNAs -- each paired either with a CFP or a RFP reporter and coupled to a mutated Cas9 enzyme. This method is reported to be more relevant to human gene modification, since it limits off-target effects.

d. Inventions, patent applications and/or licenses. *Not applicable.*

e. Other products.

- Thus far, this project has allowed us to generate several cell lines of interest such as the UT-7/Tpo cells and the UT-7 cells expressing various combinations of wildtype, or mutant, mNeonGreen-tagged Mpl with TagRFP-T-tagged calreticulin. Importantly, we have also established the genome-edited UT-7/Tpo cells expressing the calreticulin mutation of type 1 (most common mutation found in MPNs). Other genome-edited cell lines will be generated during next reporting period.
- We have collected and stored samples from over 45 MPN patients. This material is annotated for patient privacy and viable cells are in cryostorage for use in the next reporting period.

7. Participants and other collaborating organizations

a. Individuals who worked on the Project

Name:	Bridget Wilson, PhD
Project Role:	Principal Investigator
Research Identifier (eg, ORCHID ID)	orcid.org/0000-0002-3775-4450
Nearest person month worked:	1.2
Contribution to Project	Manuscript preparation, data review, project co-direction with Cleyrat, financial oversight
Funding Support	2P50GM085273-07 (Wilson) 09/01/09-07/31/2019 NIH/NIGMS "New Mexico Center for the Spatiotemporal Modeling of Cell Signaling" This grant supports a cross-campus, inter-institutional National Center for Systems Biology. UNM, Los Alamos National Laboratory and Burnham Sanford Research Institute are participating institutions. Role: Center Director P30CA118100 (Willman) 9/01/15–08/31/20 NIH/NCI NIH/NCI UNM Cancer Center Support Grant Role: Co-Leader, Translational Cancer Biology & Signaling Program R01GM100114 (Lidke) 05/01/12-02/28/17 NIH/NIGMS "Single molecule imaging to quantify FcεRI signaling dynamics." Role: co-I R01GM114075 (Bruchez, Lidke)4/1/2015-1/31/2019 NIH/NIGMS "Fluorogen activiating peptide-based FRET to quantify FcεRI activation mechanisms. Role: co-I F31CA192848 (Erasmus) 3/10/2015-3/28/2018 NIH/NCI "Validation of the pre-BCR signaling complex in pre-B ALL cell model by two-color single particle tracking and peptidomimetic inhibition." Role: Primary faculty mentor NIH R13AI126647 (Wilson) 71/2016-6/30/2017 NIH/NIAID This award provided support for the July 2016 FASEB summer conference on "IgE and Allergy: 50 Years and Onward." Role: Wilson was a conference organizer.

Name:	Cédric Cleyrat
Project Role:	Co-Principal Investigator
Research Identifier (eg, ORCHID ID)	0000-0002-1928-6497
Nearest person month worked:	4.8
Contribution to Project	Laboratory lead, including planning experiments, imaging, processing of patient samples and design of CRISPR genome editing protocols. Supervisor for Choi.
Funding Support	American Cancer Society-126768-IRG-14-187-19 (**PI: Cleyrat**) 04/2015-03/2017 Pilot study on myeloproliferative neoplasm diseases progression and leukemic transformation. Role: PI NIH NIGMS, P50 GM085273-06 (PI: Wilson) 8/2009-07/2019 Center for the Spatiotemporal Modeling of Cell Signaling Networks (STMC) *Role: co-Investigator (since 01/2015)*

Name:	Ichiko Kinjo
Project Role:	Senior postdoc
Research Identifier (eg, ORCHID ID)	n/a
Nearest person month worked:	2.4
Contribution to Project	Planning for xenograft model, flow cytometry. *Note that Dr. Kinjo's role in the next reporting period will be replaced by use of the Cancer Center Animal Model Core, on a fee-for-service basis.*
Funding Support	n/a

Name:	Eunice Choi
Project Role:	Technician
Research Identifier (eg, ORCHID ID)	n/a
Nearest person month worked:	7.2
Contribution to Project	Ms. Choi was recruited to the project based upon her experience with CRISPR and Talen genome editing. Under Cleyrat's supervision, she conducts all hands-on experiments for genome editing Baf3 and UT-7 cells, followed by cell sorting and testing. She also assists with processing of patient samples.

Funding Support	n/a

b. Changes in active other support of the PD/PI(s) and senior/key personnel during reporting period.

The following support ended during this period:

- **Wilson**, subaward PI NIH NIAID R01AI097154 (Werner) 8/1/12-31/16
  "Three-dimensional molecular tracking of IgE-FcεRI in live cells."
- **Wilson, PI,** MedImmune, LCC. Sponsored Research Agreement. 1/2012-12/2015
  "Mechanistic profiling of anti-CD22 immunotoxin for treatment of pre-B ALL"

The following support began during this period:

- Cleyrat, Cédric Center for Integrated Nanotechnology 1/2016-06/2017
  "Three-dimensional molecular tracking of EGFR in Live Renal Epithelial Cells"
- Wilson, PI P50GM085273-S1 Administrative Diversity Supplement 6/1/2016-7/31/2018

c. Partner organizations

Organization Name: Inserm UMR892/CNRS UMR6299, Centre de Recherche en Cancérologie Nantes-Angers (CRCNA), Institut de Recherche en Santé - 2 (IRS-2) - Université de Nantes
Location: Nantes, France (Sylvie Hermouet)
Partner's contribution to the project: collaboration

Organization Name: University Hospital of Nîmes, Laboratory of Clinical Cytology and Cytogenetics (Eric Carillo)
Location: Nimes, France
Partner's contribution to the project: collaboration

8. Special Reporting Requirements

a. Collaborative awards. *Not applicable*

9. Appendices.

- Abstract for American Society for Hematology Annual Meeting.

APPENDIX A

Abstract for the ASH annual Meeting (Supported in part by DOD)

In Vitro Functional Rescue of a Double MPL K39N/W272R Mutant Associated with Congenital Amegakaryocytic Thrombocytopenia (CAMT) Using CRISPR/Cas9

Cédric Cleyrat[1,2], Romain Girard[1,] Éric Jeziorski[3], Thierry Lavabre-Bertrand[4], Sylvie Hermouet[2], Serge Carillo[4] and Bridget S. Wilson[1]

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Introduction: Thrombopoietin (Tpo) and its receptor, Mpl, are the principal regulators of early and late thrombopoiesis. Mutations in MPL can drastically impair its function and be a contributing factor in chronic thrombocytosis and in congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is a rare inherited syndrome characterized by thrombocytopenia at birth, progressing to bone marrow failure and pancytopenia. The functional impact of CAMT mutations on Mpl signaling/trafficking is yet to be determined and could be relevant to multiple hematologic malignancies. Here we report unique familial cases of CAMT presenting with a previously unreported MPL mutation: T814C (W272R) in the background of the MPL K39N (Baltimore mutation), known to cause hereditary thrombocytosis (HT).

Patients: Consanguineous parents and their eldest daughter, all heterozygous for Mpl K39N/W272R, do not present any signs of disease. Their monozygotic twin daughters presented at birth with severe thrombocytopenia (platelet counts: 12,000/L and 14,000/L), low hemoglobin levels (10.4 mg/dL and 7.8 mg/dL) and very high Tpo levels (3,650 pg/mL and 3115 pg/mL). Bone marrow smears performed 19 days after birth showed severe megakaryocytopenia (only 1 megakaryocyte (MK) seen). Bone marrow colony formation assays yielded 3 and 9 MK colonies vs 84 MK colonies/105 mononuclear cells for the control, leading to a diagnosis of CAMT type I. Whole blood sequencing revealed the presence of a homozygous double MPL K39N/W272R mutation. One of the twins died after bone marrow transplant. A younger male sibling, homozygous for MPL K39N/W272R mutation, has also been diagnosed with CAMT type I.

Objectives: This study focuses on the functional characterization of the novel MPL W272R and K39N/W272R mutations and in vitro genetic engineering as a potential therapeutic option for CAMT.

Methods: Human megakaryoblastic UT-7 and murine Ba/F3 cells stably expressing human wild-type (WT) Mpl or K39N, W272R or doubly mutated K39N/W272R Mpl fused to mNeonGreen were used as models. Confocal microscopy, proliferation and surface biotinylation assays, as well as co-immunoprecipitation (co-IP) and western blotting analysis, were used to elucidate the function and trafficking of Mpl mutants. CRISPR/Cas9 genetic engineering was used to repair mutant MPL and rescue its function.

Results: Confocal microscopy shows that a significant fraction of chimeric WT Mpl protein reaches the cell surface. Significant surface expression is also noted for Mpl K39N. In contrast, the chimeric Mpl protein bearing the W272R mutation, alone or together with the K39N mutation, showed no detectable surface expression of the Tpo receptor. These results were confirmed by surface biotinylation assay. Co-expression of WT CALR fused to RFP, used as an ER marker, showed significantly higher co-localization (Pearson's R value) with mutant Mpl than with WT Mpl, evidence that the large majority of receptors were retained within the ER. We also evaluated Tpo signaling through the JAK/STAT, MAPK and PI3K pathways and Tpo-induced proliferation. Both WT and K39N-mutated Mpl were competent for signaling, while single or double mutants bearing W272R were unresponsive to Tpo. Tpo-induced signaling was partially restored via GRASP55 over-expression (forcing ER-trapped Mpl to traffic to the cell surface).

Genetic engineering performed on cells carrying the W272R mutation restored the WT sequence and the response to Tpo, with similar cell proliferation as WT Mpl cells. In addition, co-IP studies indicate that Jak2 associates strongly with WT Mpl and Mpl K39N but not with Mpl W272R.

Conclusion: We report a new mutation of Mpl (W272R) present in cis with HT-causing K39N mutation in the context of CAMT. The absence of symptoms in the Mpl K39N/W272R-mutated parents (found to be heterozygous) can be explained by the opposite (apparently neutralizing) effects of the two mutations on the trafficking and signaling of Mpl, as shown by confocal microscopy, western blotting and proliferation assays. In children homozygous for Mpl K39N/W272R, the W272R mutation prevented Mpl binding of Jak2 and expression at cell surface, rendering Tpo signaling impossible despite the presence of the K39N mutation. Function of the deficient Mpl receptor could be rescued using two separate approaches: CRISPR/Cas9 genetic engineering and GRASP55 over-expression. Cell-permeable Tpo analogs will also be tested.